Acidic compartments and rhoptry formation in *Toxoplasma gondii*

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SUMMARY

DAMP (3-(2,4-dinitroanilino)-3′amino-*N*-methyldipropylamine), which differentially accumulates in acidic compartments, was used to identify such compartments in *Toxoplasma gondii* tachyzoites at the electron microscope level. In both free tachyzoites and dividing intracellular parasites the only sites of DAMP accumulation were mature and forming rhoptries. No labelling of other secretory organelles (micronemes and dense granules), the ER, Golgi or any other membrane-bounded organelles or anything resembling a lysosomal system was observed. Labelling of the forming rhoptries was higher and more homogenous than in mature rhoptries in which labelling was confined to the expanded ends of each organelle. The acid pH-dependent accumulation of DAMP in the forming and mature rhoptries was blocked by ammonium chloride and monensin, reagents known to abolish intracellular pH gradients. Estimates of rhoptry pH, based on the level of DAMP accumulation, show that the intralumenal pH of forming rhoptries is more acidic (pH 5·5–3·5) than the mature rhoptries (pH 7·0–5·0).

Key words: *Toxoplasma gondii*, rhoptries, acidic organelles, pH, rhoptry biogenesis.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects a variety of cell types from a wide range of vertebrate hosts, including man (Joiner & Dubremetz, 1993). *In vitro* the invasive tachyzoite rapidly enters host cells and multiplies within a highly modified vacuole which does not acidify or fuse with the host lysosomal system (Sibley, Weidner & Krahenbuhl, 1985; Joiner *et al.* 1990). Once established within the host cell the parasite replicates by an unusual process of internal budding whereby 2 new daughter cells are formed within each mother cell (see Chobotar & Scholtyseck, 1982). During each round of replication the parasite manufactures 2 new conoids with the associated inner membrane complex (IMC) and microtubules and a new complement of rhoptries and secretory organelles. In addition, during the latter stages of the budding process, the conoid and part of the subpellicle cytoskeleton along with the rhoptries and micronemes in the mother cell disappear. It is still not known exactly what becomes of these structures although the implicit assumption is that they are broken down and reabsorbed by the daughter cells during the final stages of the budding process.

Apart from its mode of replication, another unusual feature of *Toxoplasma* is the absence of a morphologically identifiable lysosomal system although vesicular structures in the anterior regions of the parasite which accumulate the lysosomotropic agent, acridine orange, have been described (Norrby, Lindholm & Lycke, 1968). In eukaryotic cells, lysosomes are the terminal organelles in the endocytic pathway as well as being important in autophagic processes, metabolite storage and ion homeostasis (see Kornfeld & Mellman, 1989). Thus the lack of a lysosomal system in *Toxoplasma* raises a number of questions regarding cellular processes within the parasite. Apart from the question how the parasite recycles its apical complex and associated structures during budding, recent studies have highlighted the potential importance of acidic compartments in other aspects of *Toxoplasma* biology. First, Schwab *et al.* (1994) demonstrated that the antibiotic azithromycin accumulated within acidified organelles of the parasite and host cell but not within the parasitophorous vacuole which is not acidified. Second, Moreno & Zhong (1996) showed that tachyzoites possess a significant amount of intracellular Ca^{2+} stored in an acidic compartment(s) which they termed the acidocalcisome. It would, therefore, be of considerable interest to identify, at the ultrastructural level, the acidic compartments within *Toxoplasma*.

In the present study we have used DAMP (3-(2,4-dinitroanilino)-3′amino-*N*-methyldipropylamine), a basic congener of dinitrophenol (DNP), that differentially accumulates in acidic compartments (Anderson *et al.* 1984) to identify such compartments in *T. gondii* tachyzoites at the electron microscope level. Using this method, we found that the only

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acidic compartments in both free and intracellular tachyzoites were the mature and forming rhoptries. Estimates of rhoptry pH showed that the forming rhoptries were more acidic than mature rhoptries indicating a shift in the pH during maturation coinciding with an increase in electron density of the rhoptry contents and consistent with secretory granule formation and propeptide processing in higher eukaryotes.

MATERIALS AND METHODS

Parasites and cells

Toxoplasma gondii (RH strain) tachyzoites were maintained by serial passage in confluent monolayers of primary human foreskin fibroblasts (HFF) as described previously (Roos *et al*. 1994). Parasites were harvested shortly after complete lysis of the host cell monolayer and purified by filtration through 3 μm pore-size polycarbonate filters (Nucleopore, Cambridge, MA).

For all the experiments reported here, HFFs grown in 35 mm Petri dishes were infected as outlined below. Free parasites and host cells were cooled to 4 °C for 10–15 min, after which the medium was removed from the host cells and replaced with 1 ml of cooled parasite suspension (approximately 10^6 parasites/ml). The dishes were placed at 4 °C for 10–15 min to allow parasites to settle and attach to the host cells after which the supernatant medium and unattached parasites were aspirated and replaced with fresh medium. Infected cell cultures were incubated at 37 °C for 24 h before use.

To determine the optimal concentration of DAMP and incubation conditions required to localize acidic compartments in *Toxoplasma* tachyzoites, 24 h-infected cultures were treated with various concentrations of DAMP for up to 30 min at 37 °C and fixed and processed for electron microscopy as outlined below. In all cases the only sites of DAMP accumulation in the parasite were the mature and forming rhoptries. Based on these initial results in all subsequent experiments described below infected cell cultures and free tachyzoites were incubated with 30 μM DAMP for 30 min at 37 °C prior to fixation and processing for the immunological detection of DAMP.

To compare directly the sites of DAMP localization in both free and intracellular tachyzoites, samples were incubated in 30 μM DAMP for 30 min at 37 °C prior to fixing and processing for electron microscopy. To verify the acid pH-dependent nature of the sites of DAMP accumulation, intracellular parasites were variously treated with agents that abolish intracellular proton gradients. Infected cells were either treated with 25 mM ammonium chloride for 15 min prior to the addition of 30 μM DAMP for 30 min, or were first incubated with 30 μM DAMP for 30 min and then treated for 5 min with 1 μM monensin prior to fixation. In these experiments, to minimize possible variations, the same freshly prepared solution of DAMP was used and the samples fixed and processed in parallel.

Specimen preparation

For the localization of DAMP, both free tachyzoites and infected host cells were fixed at 4 °C in 1% glutaraldehyde (from an 8% stock solution from Electron Microscope Sciences, Fort Washington, PA) in 100 mM phosphate buffer (pH 7·2) for 1 h. The free tachyzoites were subsequently pelleted at 13000 ***g*** for 5 min. The infected fibroblasts were released from the Petri dish using a bevelled cell scraper and pelleted. All samples were dehydrated in increasing concentrations of ethanol to 70% and embedded in LR White (Electron Microscope Sciences). Polymerization was carried out at 37 °C over 5 days. Sections 50–70 nm thick were cut and collected on uncoated nickel grids.

Immunolocalization of DAMP

Sites of DAMP accumulation were detected using an antibody reactive against DNP. Sections were incubated for 2 h at room temperature in a moist chamber with anti-DNP IgG (Sigma Chemical Co., St Louis, MO.; diluted 1:100 (v/v) in PBS–glycine) followed by washing and incubation in rabbit anti-mouse IgG conjugated to 5 nm gold particles (British BioCell International, Cardiff, UK) at 1:100 dilution in PBS–glycine.

After 3 × 10 min washes in PBS, the antibody-treated grids were rinsed extensively with distilled water, air-dried and then stained with 1% uranyl acetate in 30% methanol. Sections were examined in a Philips 200 electron microscope.

As controls, sections were incubated in the second antibody-gold probe only. In addition, sections of infected cells that had not been exposed to DAMP but were fixed and processed as described above were incubated sequentially with the anti-DNP and rabbit anti-mouse-gold conjugate.

To confirm that the DAMP-containing structures were rhoptries, sections were immunolabelled with a monoclonal antibody reactive with rhoptry proteins, ROP 2,3 (Sadak *et al*. 1988) either singularly or in combination with anti-DNP antibody. For the co-localization experiments, sections were labelled sequentially with the anti-DNP and Rop 2,3 antibodies using 5 nm and 10 nm gold-conjugated secondary antibodies respectively. To rule out the possibility of the first antibody and/or gold conjugate interfering with the localization of the second primary antibody, the order of antibodies and gold conjugates was reversed.

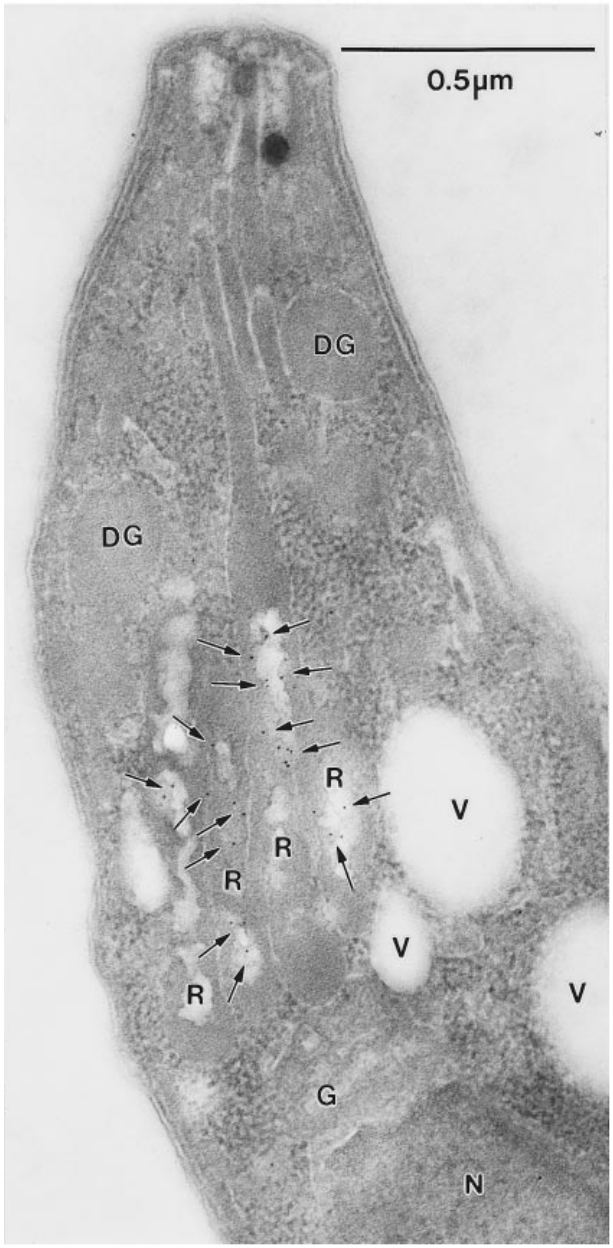

Fig. 1. Localization of DAMP in free *Toxoplasma gondii* tachyzoites was restricted to the mature rhoptries. Parasites were incubated in the presence of 30 μM DAMP for 30 min at 37 °C, fixed and embedded in LR White. Ultrathin sections were incubated with mouse anti-DNP antibodies and then with rabbit anti-mouse conjugated to 5 nm gold particles. High magnification image of the anterior end of a free tachyzoite showing labelling for DAMP only in the expanded distal region of each rhoptry (R – arrows). No labelling of the dense granules (DG), the anterior conoid region where micronemes localize, the Golgi region (G) just anterior to the nucleus (N), or with any other vacuolar organelle present in the cell (V) could be found.

Quantification of DAMP labelling and estimation of pH

To measure anti-DNP labelling of the positive compartments and over the nucleus and general cytoplasm, counts of the numbers of gold particles/μm^2 were made from a minimum of 40 non-dividing parasites and at least 25 parasites undergoing budding selected at random from each sample. For the mature and forming rhoptries counts of gold particles/μm^2 were made from profiles of individual organelles. Counts of gold particles over the surrounding resin were used as an internal control for non-specific background labelling.

Statistical analysis of the data was performed using the Student's *t*-test with a *P* value of 0·05 as the cut-off for significance.

The internal pH of acidic compartments in *Toxoplasma* tachyzoites was estimated using the method of Orci *et al*. (1986) and Andersen and Orci (1988). In brief, it was assumed that the accumulation of DAMP in these compartments was directly proportional to the H^+ concentration and that glutaraldehyde fixation and subsequent processing retained DAMP at its sites of accumulation. Under these conditions the density of gold particles due to anti-DNP binding can be used to estimate the pH of a given compartment if it is assumed that the number is proportional to the H^+ concentration.

The estimation of pH values was made using the formula: $pH = 7{\cdot}0 - \log(D1/D2)$, where 7·0 is the pH of neutrality, D1 = the density of DAMP immunogold particles in the 'acid compartments', and D2, the density of DAMP immunogold particles in a neutral compartment (background labelling over the nucleus and general cytoplasm).

To compare directly the levels of labelling between the various experimental conditions, sections of free tachyzoites, and 24 h intracellular parasites were processed at the same time using the same reagents and analysed as described above.

RESULTS

Morphological identification of acidic compartments in Toxoplasma *tachyzoites*

Examination of both free tachyzoites and replicating intracellular parasites under all the incubation conditions tried, showed that labelling for DAMP was present only associated with the mature and forming rhoptries (Figs 1–3). Confirmation that the DAMP-containing structures were exclusively mature and forming rhoptries was obtained by co-localization of DAMP and the rhoptry proteins, ROP 2,3 to the same structures (data not shown). In the dividing parasites, forming rhoptries were found within the developing daughter conoids (Figs 2 and 3) although immature rhoptries, which labelled more intensely and more uniformly than the mature rhoptries, were found associated with the mature rhoptries in the mother cell (Fig. 2E). Occasionally, in the earliest stages of daughter cell budding, gold particles were seen in the area between the Golgi complex and the

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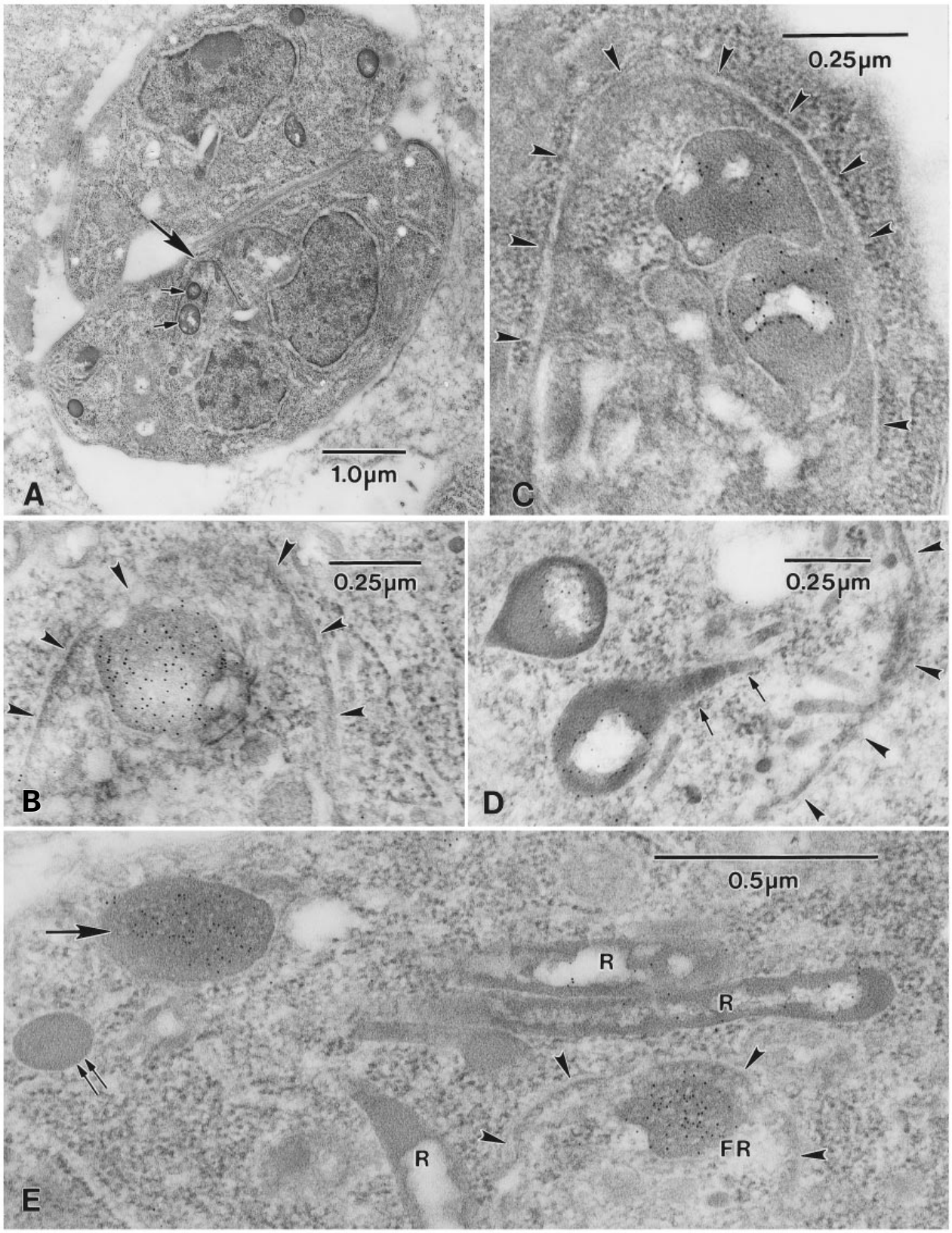

Fig. 2. In intracellular, dividing *Toxoplasma gondii* tachyzoites accumulation of DAMP is restricted to the mature and forming rhoptries. Infected cells were incubated and processed as described in Fig. 1. (A) Low-power micrograph of a pair of dividing intracellular tachyzoites showing the presence of a budding daughter cell (large arrow). The budding daughter consists of a newly formed conoid with associated inner membrane complex (IMC) and microtubules and encloses a couple of forming rhoptries (small arrows). (B–D) High magnification images of budding daughter conoids showing the range of labelling for DAMP in the forming rhoptries. Initially labelling is found over the whole of the forming rhoptry (B), although as the organelle develops labelling becomes associated primarily with

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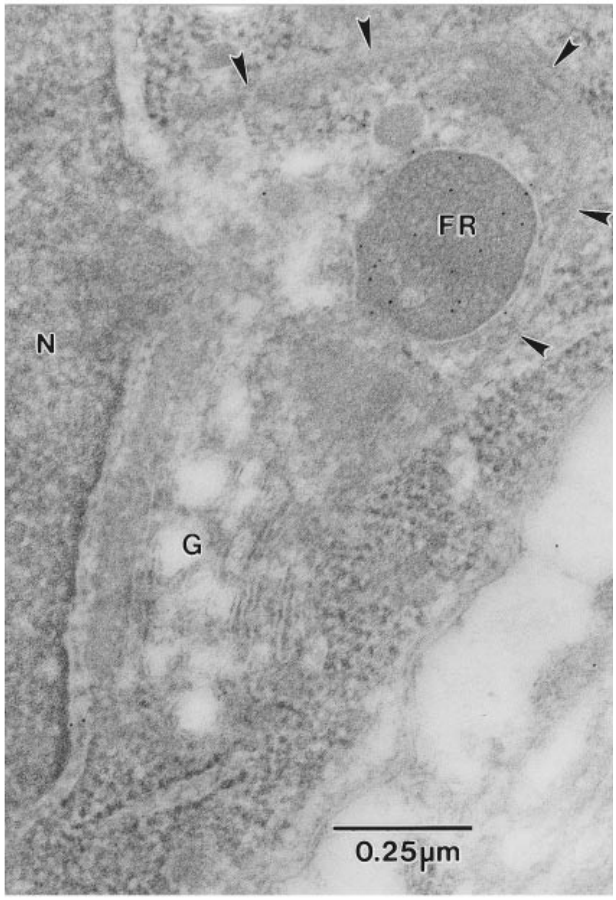

Fig. 3. High magnification view of a budding daughter parasite showing the labelling of the forming rhoptry (FR) but also the absence of labelling of the parasite Golgi (G). N, parasite nucleus. Arrowheads outline the conoid and IMC-microtubule complex of the budding daughter parasite.

forming rhoptries although the precise nature of these labelled structures was difficult to determine. However, in all cases no significant labelling of the Golgi complex was observed (Figs 1 and 3).

Labelling of the mature rhoptries in both the mother cells and in free tachyzoites was significantly lower ($P < 0{\cdot}001$) and more heterogenous than labelling associated with the forming rhoptries. In mature rhoptries, the majority of labelling was confined to the denser material around the interface between the light and dense regions of the main body of each rhoptry and no labelling of the uniformly dense neck regions of each organelle was seen (Figs 1 and 2D). The lower, more heterogenous labelling of the mature rhoptries resulted in many rhoptry profiles having no labelling and accounts for the significant proportion of rhoptries having an estimated pH of 7·0 (see below). Labelling of the forming rhoptries was initially observed throughout the denser luminal matrix and was significantly higher and more uniform than in mature rhoptries. As rhoptry formation proceeds the level of labelling over the maturing organelle becomes less dense and localized to the outer denser regions of the main body of each organelle (Fig. 2C and D).

Apart from the mature and forming rhoptries, no other sites of specific labelling were seen. Specifically, no labelling was seen in the endoplasmic reticulum (ER), Golgi complex, in the dense granules or in the micronemes located around the anterior apical complex. Furthermore, labelling was absent from the mitochondrion and multimembranous spherical body (the apicoplast; see Kohler *et al*. 1997), or from any of the vacuolar structures occasionally seen in the parasite cytoplasm (Figs 1 and 3). It should be emphasized that no labelling of anything resembling a lysosome in any free or intracellular tachyzoite was found. In addition, no labelling of the lumen of the parasitophorous vacuole was seen which is consistent with the previous observation that live *T. gondii* replicate in a non-acidic vacuole (Sibley *et al*. 1985). In the host fibroblasts, however, low levels of labelling were observed in the few multivesicular lysosomal organelles found.

In all control experiments, no specific labelling of any parasite or host cell structures was observed.

Estimation of rhoptry pH

The estimation of the pH for the mature and forming rhoptries was determined as described in the Materials and Methods section and the results shown in Fig. 4. Both the level of labelling and the estimated pH values within mature and forming rhoptries showed considerable variation, indicating a heterogeneity of pH within these organelles.

In free tachyzoites the pH of the majority of the DAMP-containing mature rhoptries was between pH 5·0 and 6·5 (Fig. 4A). However, a significant number of rhoptry profiles did not have any gold labelling and thus a proportion (25%) of the mature rhoptries had a calculated pH of 7·0. In intracellular parasites the estimated pH range of the mature rhoptries in the mother cells was slightly broader (between pH 4·5 and 6·5) with again a significant proportion (47%) of rhoptry profiles having no labelling (pH 7·0) (Fig. 4B). In contrast, the estimated pH of the forming rhoptries ranged between pH 3·5 and 6·5 with over half of the rhoptries having an estimated pH of 4·0–5·0 (Fig. 4B). Despite the

the denser regions and is absent from the lucent central areas (C, D) and from the neck of each rhoptry (small arrows in D). (E) High magnification image showing the relative levels of labelling of a forming rhoptry (FR), mature rhoptries (R) and one of the occasional immature rhoptries (large arrow) seen in the anterior regions of the mother parasite. Note that the dense granule (small arrows) shows no labelling. In (B)–(E) arrowheads outline the IMC-microtubule complex of the budding daughter parasite.

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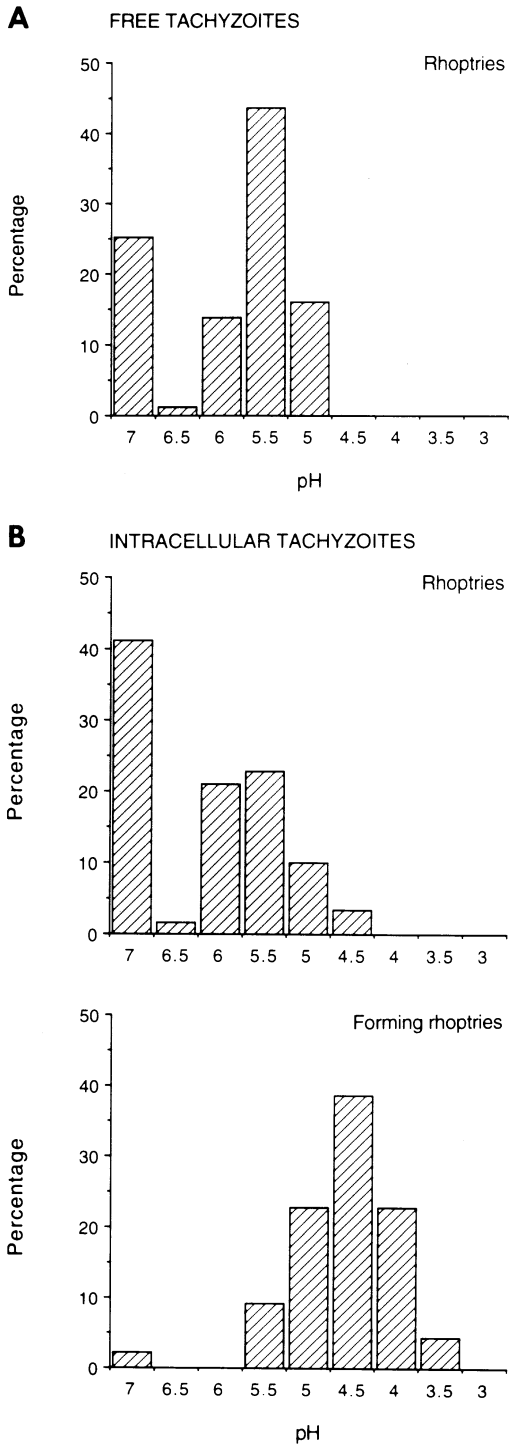

Fig. 4. Histogram of the estimated intraluminal pH of rhoptries in free (A) and intracellular (B) *Toxoplasma* tachyzoites. (A) Free tachyzoites were incubated with 30 μM DAMP and processed as described in the legend to Fig. 1. The pH was calculated as described in the Materials and Methods section, based on the accumulation of DAMP within each individual organelle. The percentage of rhoptries in each class is indicated. Total number of observations = 87. (B) Histograms of the estimated pH of rhoptries (top) and forming rhoptries (bottom) in 24 h dividing intracellular *Toxoplasma* tachyzoites. Infected cells were incubated and processed as described in the legend to Fig. 1 and the pH calculated as outlined in the Materials and Methods section. The percentage of rhoptries in each class is indicated. Total number of observations for rhoptries = 139; for forming rhoptries = 77.

heterogeneous labelling and estimated pH within the mature and forming rhoptries, it is clear that the pH of the forming rhoptries is significantly lower than in the mature rhoptries indicating that there is a shift in the pH during the maturation process with the mature rhoptries becoming slightly less acidic.

Although only a few multivesicular lysosomal organelles were seen in the host cells, estimates of their pH ranged between 6·5 and 5·3 consistent with the pH of late endosomes and lysosomes (data not shown).

To verify the acid pH-dependent nature of the intracellular sites of DAMP accumulation 24-h-old *Toxoplasma*-infected cell cultures were treated with reagents known to abolish intracellular pH gradients. When infected cells were treated with 25 mM ammonium chloride for 15 min prior to incubation with DAMP, labelling of the mature and forming rhoptries for anti-DNP-gold was significantly reduced (data not shown). In the case of the forming rhoptries treatment with ammonium chloride also caused extensive vacuolation. Similarily, when DAMP-treated cells were post-incubated with 1 μM monensin (5 min, 37 °C) labelling of the mature and forming rhoptries was again reduced (data not shown), as well as vacuolation of the forming rhoptries.

DISCUSSION

Based on the pH-dependent accumulation of DAMP, we have found that the only acidic compartments in *Toxoplasma* tachyzoites are the mature and forming rhoptries. This result is consistent with earlier light microscope observations demonstrating the presence of acidic compartments only in the anterior regions of tachyzoites (Norrby *et al.* 1968) and identifies the rhoptries as the presumable site of accumulation of toxoplasmicidal compounds such as azithromycin (Schwab *et al.* 1994). In addition, the acidic nature of the rhoptries and a previous report of Ca^{2+} being present in the rhoptries (Akao, 1981) imply that these organelles are the acidocalcisomes, the non-mitochondrial Ca^{2+}-containing acidic compartment described by Moreno & Zhong (1996). The functional significance of the rhoptries in Ca^{2+} homoeostasis in the parasite as opposed to a role in rhoptry biogenesis and secretion, however, remains to be determined. Lastly, the lack of localization of DAMP to any other vacuolar organelle(s) is also consistent with the reported absence, based on morphological and cytochemical evidence, of a lysosomal system in *Toxoplasma*. Interestingly, acid phosphatase activity (a common but not exclusive

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marker of lysosomal compartments) has been localized to the dense granules and rhoptries (Vivier & Petitprez, 1972; Metsis, Pettsersen & Petersen, 1995) although the functional significance of this observation is not known.

The acidic nature of the rhoptries in *Toxoplasma* is consistent with both the formation of secretory vesicles in eukaryotic cells in general (Mellman, Fuchs & Helenius, 1986), and with what little is known about the structure and biogenesis of rhoptries. In general, secretory vesicles are formed from clathrin-coated vesicles that bud from the trans-Golgi. During subsequent maturation the contents of the immature secretory vesicles become greatly condensed probably as a result of the acidification of the vesicle lumen due to the presence of proton pumps in the vesicle membrane. This condensation/acidification process is often accompanied by the activation of proteolytic enzymes that generate the active form(s) of the secretory molecules (Anderson & Orci, 1988; Orci *et al*. 1994; Xu & Shields, 1994; Schmidt & Moore, 1995; Anderson *et al*. 1997).

In *Toxoplasma* tachyzoites the rhoptries, micronemes and dense granules are formed *de novo* in each daughter cell during replication. While it is known that the secretory organelles of *Toxoplasma*, and apicomplexans in general, contain complex mixtures of polypeptides, some of which are partitioned to specific regions of the organelle (Sadak *et al*. 1988; Leriche & Dubremetz, 1991; Perkins, 1992; Joiner & Dubremetz, 1993; Soldati *et al*. 1995; Sam-Yellowe, 1996) as well as an array of lipids (Foussard, Leriche & Dubermetz, 1991), little is known about the dynamics of their biogenesis. In *Toxoplasma*, during rhoptry formation the expanded distal portion of each rhoptry forms before the neck region. Moreover, formation of some rhoptry proteins occurs by the processing of larger precursor molecules (Sadak *et al*. 1988). Interestingly, the lack of labelling of the neck region of the mature rhoptries with anti-DNP-gold particles may reflect differences in pH between regions within the organelle or differences in the composition of the various regions. For example, the low pH in the expanded distal parts of each rhoptry may indicate the continuing addition and processing/condensation of rhoptry proteins. In parasites fixed for conventional electron microscopy (glutaraldehyde followed by osmium tetroxide) the expanded distal part of each rhoptry has a bipartite structure with the central region being less electron dense. By contrast, the neck region of each rhoptry has a uniformly dense appearance consistent with differences in the composition of the different regions of the organelle (Soldati *et al*. 1995). Data on rhoptry biogenesis in other apicomplexans are also consistent with the sequential formation of each organelle as well as the differential incorporation of rhoptry proteins during the different stages of organelle development (Jaikaria *et al*. 1993). Furthermore, in malaria the rhoptries, micronemes and dense granules are formed sequentially during merogony (see Ward, Tilney & Langsley, 1997) implying a high degree of regulation of both protein synthesis and the packageing and targetting of materials exiting the parasite Golgi. Evidence from *Plasmodium falciparum* demonstrates that rhoptry biogenesis is controlled in part by the movement of proteins through the secretory pathway (Howard & Schmidt, 1995).

While the acidic nature of the forming and mature rhoptries is consistent with secretory granule formation, the finding that the micronemes and dense granules in *Toxoplasma* cells do not accumulate DAMP and are not acidic was surprising. Metabolic labelling and immunoprecipitation studies have demonstrated that at least 1 microneme protein is processed from a higher molecular weight precursor (Achbarou *et al*. 1991). However, since we found no anti-DAMP labelling of the micronemes the processing of micronemal proteins may have different biochemical requirements from those involved in rhoptry protein processing. At present, however, little is known about the biogenesis of these two classes of secretory organelles. The location of the micronemes at the apical end of the cell in association with the rhoptries may indicate that they are formed from Golgi-derived vesicles albeit at a later time than the rhoptries (see Ward *et al*. 1997).

The acid pH of the mature and forming rhoptries could be disrupted by treatment with ammonimum chloride and monensin. In both cases the effect was to shift the pH values of the organelles to a less acid range as well as cause morphological changes to the forming rhoptries. These observations suggest that rhoptry formation might be blocked by reagents that inhibit vacuolar acidification processes. However, examination of intracellular parasites treated with ammonium chloride, monensin and the vacuolar proton pump inhibitor, bafilomycin A1, showed that while these reagents lead to swelling of the forming rhoptries they also induced other, often extensive, structural changes. In particular vacuolation of the ER, nuclear envelope and general cytoplasm, as well as swelling of the mitochondrion was common. However, this general disruption of the parasite cytoplasm mirrored perturbations observed in the host cell implying that they represented generalized effects of the reagents and are not parasite specific.

The absence of DAMP accumulation in any structure(s) other than the rhoptries is consistent with the reported absence of a lysosomal system in *Toxoplasma*. Lysosomes are the end stage of the endocytic pathway although they also play a central role in autophagic processes, metabolite storage and ion homeostasis (see Kornfeld & Mellman, 1989). For *Toxoplasma*, evidence for endocytosis is limited (Nichols, Chiappino & Pavesio, 1994), although lysosomal-like multivesicular vacuoles are present in

a small population ($\ll 2\%$) of intracellular tachyzoites (Shaw, unpublished observation). However, while endocytosis may be limited to specific parasite stage(s) or age, the general absence of lysosomes in *Toxoplasma* raises questions regarding basic cellular processes in this parasite. In particular, it has been assumed that the conoid and rhoptries of the mother cell are re-absorbed into the parasite during cell budding. The lack of a lysosomal system would appear to rule out recycling these structures by autophagy. It is possible that the mother cell rhoptries discharge into the parasitophorous vacuole during the final stages of cell division. However, the conoid complex being a cytoplasmic structure could presumably become ubiquinated and degraded via a proteasomal system within the cytoplasm. We are currently examining the effects of protease and proteasome inhibitors on *Toxoplasma* replication and these studies will be the subject of a separate report.

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